JOURNAL OF BACTERIOLOGY, Feb. 1983, p. 1111–1113
0021-9193/83/021111-03$02.00/0

Vol. 153, No. 2

Cloning of the Aerobactin-Mediated Iron Assimilation System of Plasmid ColV

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Received 3 September 1982/Accepted 4 November 1982

The high-affinity iron assimilation system of plasmid ColV-K30 was cloned on the vector plasmid pPlac. Plasmid pABN1 was isolated by means of sensitivity to cloacin, a bacteriocin using the same outer membrane receptor as ferric aerobactin. Restriction maps were determined for this plasmid and for a subclone, pABN5. Plasmid pABN1 codes for the complete gene complex, whereas plasmid pABN5 encodes only the biosynthetic genes for aerobactin. Regulation of the uptake system by iron is retained in cloned sequences of pABN1.

Although abundant, iron is quantitatively insoluble in an aerobic environment at biological pH. This has required the evolution of special mechanisms for the solubilization and transport of the element, which is probably essential for all living cells. Iron is toxic in excess, its assimilation being regulated at the membrane level in microorganisms, plants, and animals. In the latter species, it is well established that iron is internally recycled between functional sites and storage depots.

Many species of aerobic and facultative anaerobic bacteria, as well as fungi, are known to take up iron via high-affinity pathways. This process involves the participation of low-molecular-weight, Fe(III)-specific ligands, termed siderophores, and their cognate membrane receptors. Both components of the high-affinity system are regulated, apparently coordinately, by the iron supply (5,6).

The ability to express the high-affinity system of iron uptake has been correlated with virulence for a variety of microorganisms pathogenic to animals and humans (11). Recently it has been demonstrated that it is the ColV plasmid-directed (12) synthesis in clinical isolates of *Escherichia coli* of a hydroxamate-type siderophore (7,13) that accounts, at least in part, for the invasiveness of these organisms. The particular siderophore has been identified as aerobactin (10). The ferric aerobactin receptor, which also serves as the binding site for the bacteriocin cloacin (9), has been identified as a 74 kilodalton (K) outer membrane protein (2).

To learn more about the molecular mechanism of biosynthesis and transport of aerobactin, including the regulation of these processes, we cloned the aerobactin gene complex of the ColV-K30 plasmid into a multicopy vector, pPlac.

Plasmid pColV-K30 DNA was digested to completion with restriction endonuclease *Hin*dIII to give 11 fragments ranging in size from 1.2 to 30 kilobases (kb) and summing to about 90 kb. These fragments were ligated in a 10-fold molar excess into the *Hin*dIII site of expression vector pPlac. This 2.0-kb vector plasmid is a derivative of pGL101 (8) carrying the ampicillin resistance gene of pBR322 and extending just past the origin of replication.

The ligation mixture was used to transform *E. coli* K-12 294 (*endA hsdR thi pro*; obtained from M. J. Chamberlin). Transformed *E. coli* 294 cells were selected by ampicillin resistance. In a second, negative selection, ampicillin-resistant colonies were then screened by replica plating onto LB plates (4) containing ampicillin and cloacin, the latter serving as an indicator for the expression of the ferric aerobactin/cloacin receptor. Of 500 ampicillin-resistant transformants a single colony was found that had acquired cloacin sensitivity. This transformant contained an 18.3-kb plasmid which had the *Hin*dIII B fragment of pColV-K30 inserted into the single *Hin*dIII site of pPlac. The restriction map of the recombinant plasmid, designated pABN1, is shown in Fig. 1. These data were derived from single and double digests of pABN1.

Plasmid pABN1 was doubly digested to completion with restriction endonucleases *Hin*dIII and *Eco*RI. The 95 base-pair *Hin*dIII-*Eco*RI *lac* promoter fragment of pPlac was removed by gel filtration through Sepharose Cl-2B-300, and the resulting digest was religated and used for transformation of *E. coli* 294. An ampicillin-resistant transformant carrying an 8.7-kb recombinant plasmid, designated pABN5, was isolated. This plasmid consists of the *Hin*dIII-*Eco*RI B fragment of pABN1 and of the vector pPlac without

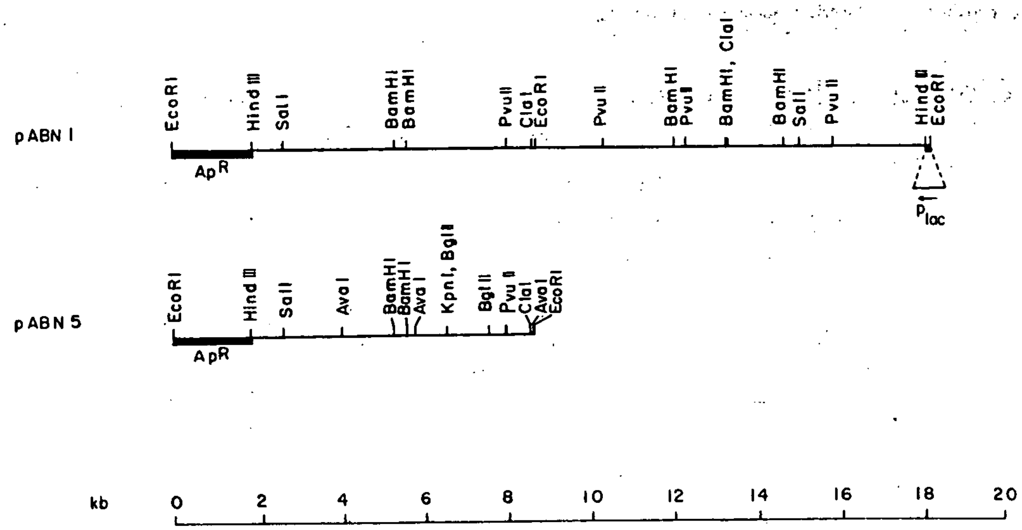

FIG. 1. Restriction maps pf pABNl and pABN5. The arrow specifies the direction of transcription from the *lac* promoter.

the *Hin*dIII-*Eco*RI promoter fragment. The restriction map, with some additional restriction sites determined, is also shown in Fig. 1.

In the same way, plasmid pABN3 was constructed and shown to contain the *Hin*dIII-*Eco*RI A fragment of pABN1 and the 1.9-kb *Eco*RI-*Hin*dIII pPlac vector fragment (not shown in Fig. 1).

To characterize the cloned sequences, *E. coli* BN3040 Nal^r (*entA cir*) (3) was transformed with DNA of plasmid pABN1. This strain has a genetic background suitable for the study of cloned sequences of the ColV-K30 iron uptake system. It is *entA* and is hence defective in the synthesis of enterobactin (enterochelin), the chromosomally coded, high-affinity iron uptake system common to most enteric bacteria. BN3040 Nal^r is also *cir*, a circumstance resulting in the absence of the Cir protein, which comigrates on gels with the 74K ferric aerobactin receptor (2).

The expression of the 74K protein in BN3040 Nal^r(pABN1) was comparable to that observed for BN3040 Nal^r(pColV-K30) when both strains were grown at low iron concentrations (Fig. 2). In iron-rich medium, such as L broth, the 74K protein was fully repressed in BN3040 Nal^r(pColV-K30) and was present in only minute amounts in BN3040 Nal^r(pABN1). Similarly, supplementation of the minimal medium with 20 μM ferrous sulfate repressed to a marked degree the expression of the 74K protein in BN3040 Nal^r(pABN1).

These results indicate that iron-dependent regulation of the ferric aerobactin receptor gene is retained in the cloned sequences. The high copy number may be responsible for the residual low expression of the 74K protein under high-iron conditions.

Plasmid pABN1 contains not only the gene for the ferric aerobactin/cloacin outer membrane receptor, but also the gene complex for aerobac-

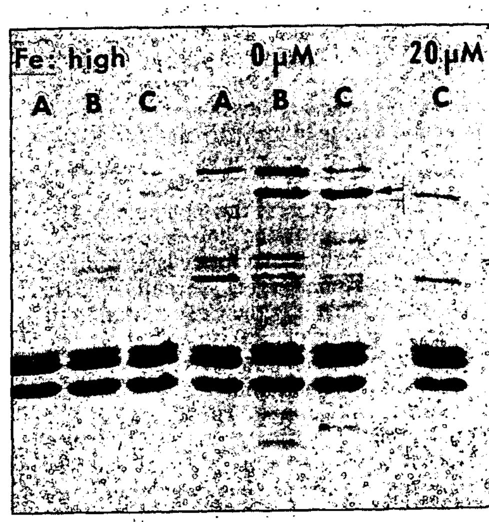

FIG. 2. Outer membrane protein profiles in high-iron medium (L-broth) and in low-iron media (Tris-succinate) with and without 20 μM ferrous sulfate. (A) BN3040 Nal^r; (B) BN3040 Nal^r(pColV-K30); (C) BN3040 Nal^r(pABNl). Location of the 74K ferric aerobactin/cloacin receptor protein is indicated by the arrow.

tin biosynthesis. This was demonstrated by use of both biological (*E. coli* LG1522) and chemical (1) assays for aerobactin with supernatants of *E. coli* 294(pABN1). Aerobactin was detected by a nutrient agar–200 μM α,α′-bipyridyl plate assay with *E. coli* LG1522 carrying the ColV-K30 *iuc* plasmid (13). This mutant plasmid is defective in biosynthesis, but not in uptake, of ferric aerobactin. Purified aerobactin and the supernatant from the *E. coli* 294 culture were used as controls. The *E. coli* 294 supernatant gave no response, and the supernatant from both low- and high-iron-grown cultures of *E. coli* 294(pABN 1) gave the same positive response as purified aerobactin. The hydroxamate assay gave the same result. Addition of 20 μM iron to a nutrient broth culture repressed aerobactin production, based on the cell density measured from absorbance at 650 nm, by about 50%.

Derivative plasmid pABN5 still carries the gene complex for aerobactin biosynthesis, as determined by a hydroxamate assay and an aerobactin bioassay with culture supernatants of *E. coli* 294(pABN5). However, as revealed by lack of sensitivity to cloacin, pABN5 does not express the 74K protein, which we believe to be at least a component of the ferric aerobactin receptor. The regulation by iron of aerobactin expression in pABN5 is still under investigation. A consideration of the phenotypes of *E. coli* 294(pABN1) and *E. coli* 294(pABN5) taken together with our finding that *E. coli* 294(pABN3) does not synthesize aerobactin and is cloacin insensitive suggests that the gene for the 74K receptor overlaps the *Eco*RI site of the pABN1 insert.

The complete gene complex for the iron uptake system of the ColV-K30 plasmid probably consists of at least five or six genes, considering that aerobactin synthesis must proceed from citrate and lysine; at least one additional gene must be required for the 74K outer membrane protein. Since all of these genes are coordinately controlled by iron, they probably exist as an operon. The latter is apparently contained within the cloned 16.3-kb fragment of pABN1.

Future experiments on the molecular genetics of pABN1 and pABN5 will focus on the organization and sequence of the gene cluster, with special emphasis on the mechanism(s) of its regulation by iron.

This work was performed under the tutelage of R. Tjian. M. Learned provided advice, and Peter H. Williams furnished the ColV-K30 plasmids.

Financial support was provided by Public Health Service grants AI04156, AM17146, and ES070705 from the National Institutes of Health and by National Science Foundation grant PCM 78-12198.

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CODEN: JOBAAY 153 (2) 581–1123 (1983)
ISSN: 0021-9193

VOLUME 153 • FEBRUARY 1983 • NUMBER 2

Journal of Bacteriology

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Published monthly by the American Society for Microbiology